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Growth factors may play an important role in the pathogenesis of breast cancer. The MAP kinase pathway may be essential for transformation caused growth factors. Thus, the purpose of this study is to determine if MAP kinases may be overexpressed or activated in breast cancer. About one-third of the breast cancer cell lines tested contained changes in the amounts of MAP kinases, although there was not one consistent pattern of alteration from the normal expression. An alteration in amount of any of the enzymes may be an indication of altered regulation of the pathway in the affected cells. Future experiments to further address this issue will focus on analyzing activation state and correlating changes in amounts of MAP kinases with stage of tumor. Newly available antibodies allow measurement of activated MAP kinases directly in cell lysates. These antibodies are being evaluated for use in immunocytochemistry of normal mammary epithelial cells and breast cancer cell lines. If these antibodies prove useful for immunocytochemistry, in the future tumors will be obtained from Dr. Gazdar of this institution so that activation state of MAP kinases can be measured in situ.

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Melanie H Cobb 10-10-96

PI - Signature Date

Melanie H. Cobb, Ph.D.

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(5) INTRODUCTION

Breast cancer is the most common cancer of women (1). Although therapies have been developed that are effective in selected subgroups of patients, these therapies have not had a great impact on breast cancer mortality rates. To develop new and more effective therapies for breast cancer it will be important to understand the pathogenesis of breast cancer in more detail. Breast cancer cells express tyrosine kinase receptors and may be induced to proliferate by their cognate growth factors (1). The role of growth factors in human breast cancer is further supported by the finding that the *erb*B2 gene is amplified in 10-30% of breast cancers (2-10). The presence of this amplified gene correlates with a poor prognosis (2-10). Overexpression of ErbB2 in NIH 3T3 cells induces transformation, and expression of a mutant ErbB2 in transgenic mice results in development of adenocarcinomas of the breast in 100% of females (11-13). These data strongly support the conclusion that breast carcinoma cells are responsive to growth factors and support the hypothesis that these factors may play an important role in the pathogenesis of breast cancer.

Ras is an important signal transducer in the actions of tyrosine kinase receptors like those for ErbB2 and insulin. Activation of Ras leads to the activation of several downstream signaling pathways. Among these the best understood is the MAP kinase pathway (14). The GTP-bound form of Ras interacts with the protein kinase Raf, which, like Ras, is a proto-oncogene. The binding of Raf to Ras is thought to target Raf to the membrane where its protein kinase activity is increased. The enzymes activated by Raf, MAP kinase kinases or MEKs 1 and 2 (15), phosphorylate and activate the MAP kinases, ERK1 and ERK2. The MAP kinases ERK1 and ERK2 are pleiotropic regulatory enzymes activated in most if not all cell types by several of numerous hormones and growth factors. They phosphorylate many substrates, including certain other protein kinases and transcription factors, and have important growth regulatory functions. This pathway has also been implicated in the transforming activity of small t antigen (16).

Interfering with the functions of ERK1 and ERK2, using catalytically defective mutants of them, blocks 1) the actions of the oncogenic forms of Ras (17) and Raf in fibroblasts, 2) the transforming potency of small t antigen in CV-1 cells (16), and 3) cell proliferation induced by EGF (16). Constitutively active forms of the MAP kinases have not been identified either by genetic selection or by mutagenesis strategies (14). However, since this proposal was submitted, it has been shown that activated mutants of MEK1 transform fibroblasts in culture, cause their growth in soft agar, and result in the formation of tumors in nude mice (18). MEK1 is one of the major targets of Raf, but it is probably not the only Raf target; thus, transformation induced by activated MEK1 is likely due to a subset of the potential actions of Raf. As the MAP kinases are the only known substrates of MEK1, it is believed that activated MEK1 transforms by activating MAP kinases. These findings support the concept that activation of MAP kinase leads to cellular transformation. Therefore, we propose that this pathway may be altered in breast cancer, perhaps increasing sensitivity to growth factors and that the activity of this pathway may be critical for the proliferation of the cells.

Given these critical role of tyrosine kinase receptors in breast cancer and the recent results with activated MEK1, the original purpose of this research was to: 1) measure the amounts of MAP kinases and MEKs in normal breast tissue and a series of cells from malignant breast; 2) measure the sensitivity of MAP kinases in these cells to insulin, serum, and EGF; 3) determine if activated MEK1 will transform cells from normal breast tissue; and 4) determine if catalytically defective mutants of MAP kinases block proliferation of breast cancer cells. The methods, described in more detail below, are primarily immunoblotting and activity assays developed in our laboratories, and morphological transformation assays. Newly available antibodies, described below, have caused us to amend our original goals to include immunocytochemistry of freshly isolated tumors.

(6) BODY

Experimental Methods

Immunodetection of enzymes of the MAP kinase pathway. The amounts of the MAP kinases present in the normal and malignant cells have been identified by immunoblotting with antibodies to ERK1 and ERK2 (19). We have generated antibodies to both isoforms that will detect both proteins cleanly in whole cell lysates by immunoblotting. ERK1 and ERK2 each undergo a shift in electrophoretic mobility when they are in the active forms. Thus, a lysate containing only inactive ERK1 and ERK2 will display two immunoreactive species, ERK2 at ~41 kDa and ERK1 at ~43 kDa. If the proteins are both fully active, two bands at ~42 kDa and ~44 kDa will be present. In a lysate containing a mixture of active and inactive ERKs, all four bands will be present and resolved. The differences in mobility are easily detected by comparison to a standard.

Measurement of MAP kinase activity. Antibodies that selectively recognize the active forms of ERK1 and ERK2 were used for immunoblotting to detect the possibility of constitutive activation of ERK1/ERK2 in cell lines derived from tumors. Because these antibodies do not recognize 100-fold excess of the inactive kinases, they can reliably detect even very small amounts of activated ERK1 and ERK2 in whole cell lysates and we tested them for for immunofluorescence as described under results of task 2 and will now proceed to test them for immunocytochemistry. We believe the methodology can be developed. Immunocytochemistry will be performed on freshly isolated tumors to determine the activation state of the MAP kinases in situ. This will allow us to determine if their is a relationship between tumor grade and MAP kinase pathway activity.

Results and future directions

Due to considerations described in last year's progress report, the tasks redefined as our statement of work are as follows:

Task 1. **Immunodetection of enzymes of the MAP kinase pathway**
Task 2. **Assessment of MAP kinase activities in tumor cells.**
Task 3. **Analysis of MAP kinases and their activities in situ in breast tumors and preneoplastic states**

Progress will be described as it relates to these three tasks.

Task 1. **Immunodetection of enzymes of the MAP kinase pathway**
Task 2. **Assessment of MAP kinase activities in tumor cells.**

In the second year of this grant, immunoblotting methods were used to assess both amounts of (task1) and activities of (task 2) ERK1 and ERK2.

Examination of the MAP kinase pathway in cells from nonsmall cell lung carcinomas (nSCLC). Ten nSCLC cell lines have been studied to probe the possible role of the MAP kinase pathway in this type of cancer. Some of the cell lines expressed mutated forms of Ras. There was a consistent trend indicating a large increase in expression of ERK1 and a marked activation in a large proportion of the cells.

Examination of the MAP kinase pathway in cells from human breast tumors and normal mammary epithelium. We have examined MAP kinase amounts in a number of nonimmortalized lines from normal breast and breast carcinoma and lines from normal and malignant tissue that have been immortalized in the laboratory. Unlike normal bronchial epithelium, normal breast tissue has nearly equal amounts of ERK1 and ERK2. About one-third of the breast cancer cells contained changes in

the amounts of these MAP kinases. Again, unlike in nSCLC, there was not one consistent pattern of alteration from the normal expression of ERK1 and ERK2. In some lines there was relatively more ERK1 and in some there was relatively more ERK2. An alteration in amount of any of the enzymes may be an indication of altered regulation of the pathway in the affected cells. However, the marked variation makes it difficult to form an initial hypothesis about the role of the cascade in breast cancer. One question that we will address by looking at fresh samples rather than cell lines is are their consistent changes in amounts of MAP kinases in tumors or are the changes we see in cultured lines a consequence of their growth in culture. To focus on the activation state of the MAP kinases, we used the active ERK1/ERK2 antibody. Immunoblots showed that ~ half of the lines displayed activated ERK2.

Our current interpretation is that the kinases in the pathway are more highly activated by growth factors in the malignant cells, suggesting that the kinases are involved in enhanced growth factor sensitivity. Again, we plan to verify this hypothesis by examining tumors with antibodies that selectively detect active enzymes. Whether activation of this cascade is required for progression to the malignant phenotype will be examined by future experiments using dominant negative ERK mutants or activated MEK1 mutants.

Task 3. Analysis of MAP kinases and their activities in situ in breast tumors and preneoplastic states

To evaluate the possibility of using antibodies to detect activation states of ERKs in tissue, we have used the antibodies for immunofluorescence on fibroblasts. First we wished to determine if we could see differences in immunofluorescent signal in serum-deprived compared to serum- or growth factor-treated cells and to assess the sensitivity of the antiactive ERK antibodies. These antibodies were provided prior to their widespread commercial availability, so that we could evaluate their usefulness. We have used several batches of antibodies and have identified affinity purified antibodies from one rabbit that readily detect differences in activation state of ERKs in fixed cells. We are in the process of adapting them for tissue immunocytochemistry.

Concerning the original tasks 2-4. Of the originally proposed tasks # 2-4, to examine effects of activating or inhibiting MAP kinases on tumor proliferation, etc., based on results of task 1, there is no logical basis for performing the future experiments on the cell lines presently on hand. The marked variation makes it difficult to form an hypothesis about the role of the cascade in breast cancer. Because of that variation in behavior of the cell lines, it seemed apparent that only actual tumor samples would suggest if there was any significant component of breast cancers that might relate in a meaningful way to the MAP kinase pathway. Nevertheless, we did analyze a large series of breast cancer and other tumor-derived cell lines for the presence of a regulatory subunit of phosphatase 2a, which has been implicated in inactivation of MAP kinases in transformed cells, and for the activation state of the MAP kinases, using antibodies that selectively immunoblot the activated forms. Our original analysis of MAP kinase activities in these lines, based on in vitro kinase assays which are more subject to misinterpretation, was inconclusive. A table of these studies and a summary of the results are included. In anticipation of performing the originally proposed tasks we have prepared and obtained the complete group of recombinant adenoviruses that would have been required, including ones that express wt and catalytically defective ERK2. These have been tested in three cell lines to confirm that they have the expected properties.

Our comparison of activities in small cell and nonsmall cell lung cancer cell lines was valuable for this study, because it shows that consistent changes in amounts and behavior of MAP kinases can be expected in certain tumor-derived cell types and that such changes depend on cell type. For example, in cell lines from nonsmall cell lung cancers, there is a consistent increase in the amount of

ERK2. This type of consistent change allows experiments to be performed in a meaningful context. In the breast cancer cells, all possible combinations of behavior were detected. The amounts of either the MAP kinases ERK1 or ERK2 went up or went down and in some, but not all, cases activities were elevated in a manner apparently not related to enzyme amounts. The results from the breast cancer cells indicate that important variables are not controlled that relate to the cell lines themselves.

(7) CONCLUSIONS

About one-third of the breast cancer cells contained changes in the amounts of MAP kinases, although there was not one consistent pattern of alteration from the normal expression. An alteration in amount of any of the enzymes is consistent with altered regulation of the pathway in the affected cells. Approximately half showed an increase in ERK2 activity, which suggests that the enhanced proliferation of the cells may relate to increased MAP kinase activity. Future experiments will focus on correlating MAP kinase activities with stage of tumor by immunocytochemistry in tumors obtained from Dr. Gazdar of this institution for this analysis. A publication concerning the work on MAP kinase amounts and activities is in preparation.

Western Result Summary

Cell Type	Activated ERK 1/2 (Ab)	ERK 1/2 (Y691)	PP2A (Ba)
NSCLC	8/8; 5/8 ERK2>ERK1, 1/8 only ERK2	8/8; 1/8 ONLY ERK2	8/8.
SCLC	1/14 (ERK2>ERK1)	14/14	14/14
Breast	10/17 (ERK2>ERK1)	17/17	17/17
V trans. B cells	0/2		

Western Blot

Lysate Number	Cell Lysate	Activated ERK 1/2 (Ab)	ERK 1/2 (Y691)	PP2A (Ba)
YE001	H358	yes (faint)	yes	yes
YE017a	H157	yes (ERK 2 > ERK 1)		
YE020b	BL-5	no		
YE023a	BL1770	no		
bbp3	H524	no	yes	yes
bbp14	SCC1187	no		
bbp15	HTB131	no		
bbp16	HTB22	no		
bbp17	HTB129	yes (ERK 2, faint)		
bbp18	HTB26	yes (ERK 2 > ERK 1)		
bbp19	HTB121	no	yes	yes
bbp20a	SCC38	yes (ERK 2 > ERK 1)		
bbp20b	SCC38	yes (ERK 2 > ERK 1)		
bbp21a	HTB24	yes (ERK 2 > ERK 1)		
bbp21b	HTB24	yes (ERK 2 > ERK 1)		
bbp22a	HTB130	yes (ERK 2, very faint)		
bbp22b	HTB130	yes (ERK 2, very faint)		
bbp23b	HTB132	yes (ERK 2 > ERK 1)	yes	yes (faint)
bbp24a	HTB133			
bbp24b	HTB133	yes (faint)		
bbp25b	HTB19	no	yes	no
bbp26b	HTB23			
bbp27a	HTB27			
bbp27b	HTB27			
bbp28b	HTB20			
bbp29a	HTB25			
bbp29b	HTB25	yes (ERK 2 > ERK 1)		
bbp30b	H345	no	yes	yes
bbp31b	H220	no	yes	yes
bbp32b	H592	no	yes	yes
bbp33b	H748	no	yes	yes
bbp34b	H2198	yes (ERK 2, faint)	yes	yes
bbp35b	H738	no	yes	yes
bbp36b	H1688	no	yes	yes
bbp37b	H889	no	yes	yes
bbp38b	H1607	no	yes	yes
bbp39b	H1994	no	yes	yes
bbp40b	H2028	no	yes	yes
bbp41b	H740	no	yes	yes
bbp42b	H1450	no	yes	yes
	H1334 no stim	yes (faint)	yes	yes (faint)
	H1334 30' stim	yes	yes	yes
11/15/95	NHBE no stim	ERK 2 (faint)	yes	yes
11/15/95	NHBE 24 hr stim	ERK 2 (+faint ERK 1)	yes	yes (faint)

Western Blot

	H1792 0'stim	yes (ERK 2 only)	yes	yes
	H1792 30' stim	yes (ERK 2 > ERK 1)	yes	yes
	H1466 0' stim	yes (ERK 2 > ERK 1)	yes	yes
	H1466 30' stim	yes (ERK 2 > ERK 1)	yes	yes
11/17/95	NHBE no stim	no	no	yes
11/17/95	NHBE 24 hr stim	yes (ERK 2 > ERK 1)	yes	no
	PC12 NGF stim	yes	yes	yes
	PC12 no stim	yes (very faint)	yes	yes

HTBs and SPCs are breast cancer cells. NHs are normal epithelium.

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